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METHOD FOR PRODUCING MONOGLYCOSIDATED FLAVONOIDS

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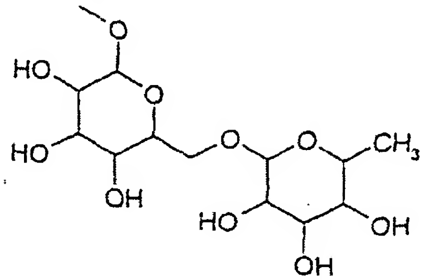
(57) Abstract: The invention relates to a method for producing monoglycosidated flavonoids by enzymatic hydrolysis of rutinosides, using an enzyme immobilized on a carrier for the enzymatic hydrolysis. The inventive method reduces the costs for the enzymes, and simultaneously provides for a high degree of automation associated and an optimized space/time yield.

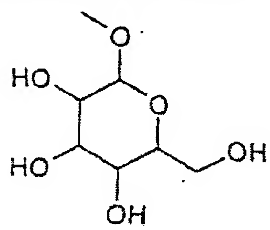
The present invention concerns a method for the production of monoglycosidated flavonoids by enzymatic hydrolysis of rutinosides. The rhamnose residue of rutinoside is enzymatically cleaved.

In the context of the present invention, compounds that contain a sugar-free component, to which a residue of the formula (I)



(I)

is bonded via a glycoside bond, are referred to as rutinosides. For example, rutinosides are flavonoids with the bisglycoside unit shown in formula (I). Rhamnose and/or the corresponding glucopyranosides can be obtained from rutinosides. Glucopyranosides are derived from rutinosides in that they contain, instead of the residue of formula (I), a residue of formula (I*)

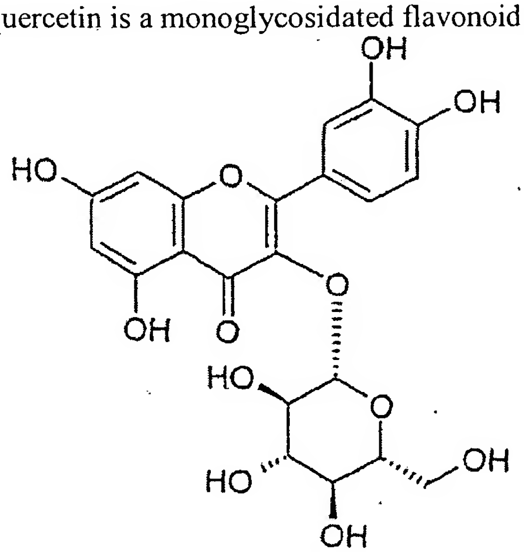


(I*)

bonded to the sugar-free component. For example, both rhamnose and isoquercetin can be obtained from rutin.

Rhamnose is a monosaccharide, common in nature, but mostly present in only limited amounts. Important sources of rhamnose are the glycoside residues of natural flavonoids, like rutin, from which rhamnose can be obtained by glycosidic cleavage. Rhamnose plays an important role as a starting material for preparation of artificial flavorings, like furaneol.

Isoquercetin is a monoglycosidated flavonoid of the following structural formula (II)



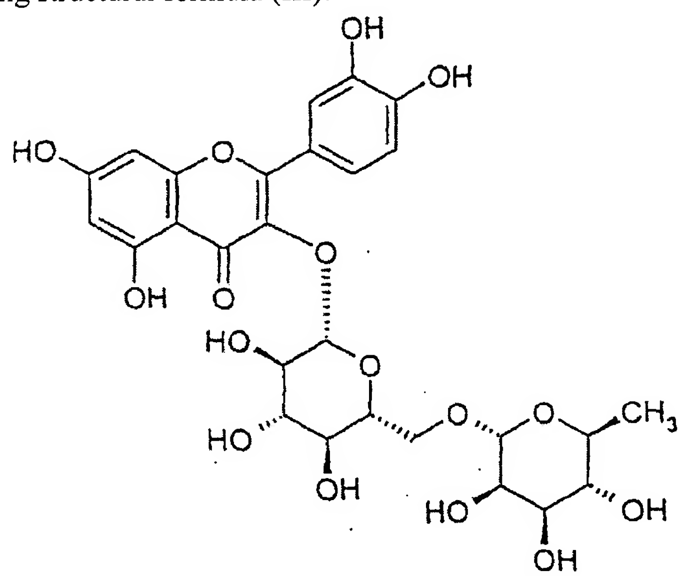
(II)

Glycosides of flavones, to which the basic framework of flavone (2-phenyl-4H-1benzopyran-4-one) is common, are referred to as flavonoids (Latin flavu = yellow), which are pigments prevalent in plants.

The sugar-free component of flavonoids is the so-called aglycone. Isoquercetin, for example, is a glycoside of the aglycone quercetin (2-(3,4-dihydrophenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one), which differs from flavone by the presence of five hydroxyl groups. In quercetin, the carbohydrate group glucose is bonded to the hydroxyl group in position 3 of quercetin. Isoquercetin is referred to as quercetin-3-O-β-D-glucopyranoside or 2-(3,4-dihydroxyphenyl)-3-(β-D-glucopyranosyloxy)-5,7-dihydroxy-4H-1-benzopyran -4-one. However, it is also known as hirsutrin.

Flavonoids and flavonoid mixtures are used, for example, in the food and cosmetics industries, and are gaining increasing significance there. Monoglycosidated flavonoids, in particular, like isoquercetin, are characterized by good absorption in the human body.

An example of a naturally occurring flavonoid with a bisglycoside unit is rutin, which has the following structural formula (III):



(III)

Rutin, like isoquercetin, is also a glycoside of the algycone quercetin, in which the carbohydrate residue rutinose is bonded to the hydroxyl group in position 3 of quercetin. The carbohydrate residue in rutin consists of a glucose linked in the 1 and 6 position and a terminally bonded rhamnose or 6-deoxymannose unit. Rutin is referred to as quercetin-3-O-β-D-rutinoside or 2-(3,4-dihydroxyphenyl)-3-{[6-O-(6-deoxy-α-mannopyranosol)-β-D-glucopyranosyl]oxy}-5,7-dihydroxy-4H-1-benzopyran-4-one. However, it is also known as sophorin, birutan, rutabion, taurutin, phytomelin, melin or rutoside.

Rutin forms pale yellow to greenish needles with three molecules of water of crystallization. Anhydrous rutin has the property of a weak acid, turns brown at 125°C and decomposes at 214-215°C. Rutin, which occurs in many plant species (frequently accompanying vitamin C), was isolated in 1842 from garden rue (*Ruta graveolens*) and occurs in citrus species, in yellow pansy, forsythia, acacia species, different *Solanum* and *Nicotiana* species, linden blossoms, St. John's wort, tea, etc. Rutin can also be obtained from the leaves of buckwheat and the East Asiatic dye drug Wei-Fa (*Sophora japonica*, Fabaceae), which contains 13-27% rutin.

It is desirable to produce both rhamnose, as well as monoglycosinated flavonoid, from natural raw materials, for example, from flavonoids with a biglycoside unit. In this context, cleavage of rutinosides to rhamnose and the corresponding glucopyranosides is of interest.

Enzymatically catalyzed preparations of rhamnose are described in the literature. For example, a method for production of L-rhamnose is described in EP-A-0317033, in which the rhamnoside bonding of glycosides that keep rhamnose bonded in the terminal position is achieved by enzymatic hydrolysis. During this cleavage, the substrate is ordinarily present as a suspension in an aqueous medium. However, these reactions are generally not very selective. For example, a mixture of the two monosaccharides glucose and rhamnose is often formed because of the biglycoside structure of the carbohydrate residue in rutin. In addition, mostly high percentages of aglycone quercetin, as well as additional undesired by-products, are formed.

Enzymatically catalyzed cleavages of rutin are also described in JP-A-01213293. However, such reactions performed in aqueous media are generally not very selective either.

The presently described methods use the enzyme in solution, i.e., in native form. The enzyme is then directly added to the reaction solution. Although these methods can be performed on a laboratory scale, they are not practicable for industrial use, since the enzyme cannot be recovered for reuse from the reaction solution. Single use of this expensive enzyme, however, is not economical during industrial use.

It is known that enzymes are industrially employable, if they are bonded to a carrier. This method is referred to as immobilization. All enzymes "... that are found in a state that permits their reuse" are subsumed under the term "immobilized enzymes" by the European Federation of Biotechnology (1983) (Helmut Uhlig, Technical Enzymes and Their Use, Carl Hanser Verlag, Munich/Vienna 1991, page 198). Despite this advantage, however, immobilization is not suitable for all enzyme processes and has thus far gained only limited acceptance. In particular, only two immobilized enzymes find industrial use: glucose isomerization with immobilized glucose isomerase, and penicillin-G cleavage with immobilized penicillin amidase. Immobilized enzyme methods often cannot compete relative to the free enzymes or chemical methods. Often the enzymes or reaction conditions are also unsuitable for immobilization. Consequently, there is no universal method for immobilization and each enzyme must be considered individually.

For example, solubility problems occur in rutinosides in aqueous systems, as are important for functional capability of enzymes, the rutinosides serving as substrate during enzymatic hydrolysis. This reaction is therefore preferably performed with a supersaturated substrate solution, i.e., as rutinoside suspension. In a supersaturated solution, in which the substrate is present as a solid, however, the use of an immobilization technique is impossible. There is no selectivity between the raw material and the product particles and the solid-bonded enzyme.

It is therefore the task of the present invention to provide a method for the production of monoglycosidated enzymes that can be used on an industrial scale, in which high enzyme costs are avoided and a high degree of automation, connected with a high space/time yield and high productivity and selectivity, is reached simultaneously. This task is solved by a method for the production of monoglycosidated flavonoids by enzymatic hydrolysis of rutinosides, in which an enzyme immobilized on a carrier is used for enzymatic hydrolysis.

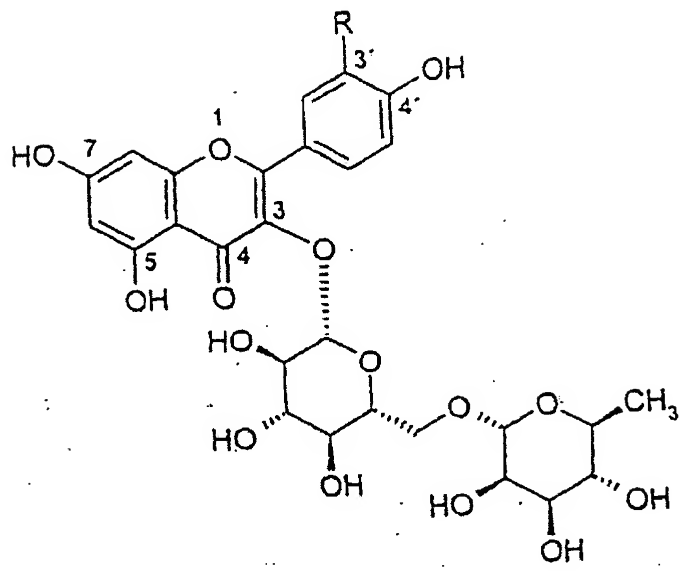
It was surprisingly found that, despite limited solubility of rutinosides, enzymatic hydrolysis is possible with an immobilized enzyme. By immobilization, the method can be performed continuously or batch-wise with high efficiency, in comparison with the reaction with native enzyme. The method according to the invention is characterized, in particular, by the fact that high automation of the entire process, including recycling of the solvent, as well as monitoring of enzyme activity, is possible.

Figure 1 shows, as an example of the method according to the invention, continuous production of isoquercetin from rutin.

Appropriate rutinosides for the method are those containing a sugar-free component or aglycone, a 2-phenyl-4H-1-benzopyran-4-one basic substance, carrying a residue of formula (I) in position 3, and whose phenyl groups, apart from position 3, can also be mono- or polysubstituted with –OH or $-O(CH_2)_n$-H, in which n denotes 1-8. n preferably denotes 1.

Substitution of the 2-phenyl-4H-1-benzopyran-[4]one basic substance with –OH and/or $-O(CH_2)_n$-H preferably occurs in positions 5, 7, 3' and/or 4'.

Rutinosides, represented by formula (A), are used with particular preference:



(A)

in which R represents H, OH or OCH_3.

The compound in which R represents H is referred to as kampferol rutinoside; the rutinoside in which R represents OCH_3 is called isorhamnetin rutinoside. The compound in which R denotes OH is called rutin. As a result, according to the method of the invention, rhamnose and kampferol glycoside can be obtained from kampferol rutinoside, rhamnose and isoquercetin from rutin and isorhamnetin glycoside from isorhamnetin rutinoside.

The rutinoside rutin is used with particular preference.

As starting material for the method according to the invention, the rutinosides can be used in pure form, or also as mixtures of rutinosides. The rutinosides can also be contaminated with other flavonoids or residues from rutinoside production, without adversely affecting the reaction.

Ordinary hydrolases can be used as enzyme for enzymatic hydrolysis of rutinosides, which are capable of cleaving the rhamnose residue of rutinosides. Hydrolases obtained from the strain *Penicillium decumbens* are preferably used. α-L-rhamnosidases are used as enzyme with particular preference, since these have high selectivity for hydrolysis of the rhamnose residue. Appropriate α-L-rhamnosidases include hesperidinase, naringinase, as well as those described in Kurosawa et al. (1973), J. Biochem., Vol. 73: 31-37. It is particularly preferred to use the enzyme hesperidinase.

Both the rutinosides and enzymes for the method according to the invention can be obtained as commercial products. It is also possible to recover or produce the initial substances and enzymes according to generally known methods.

The enzyme is immobilized on an appropriate carrier. Ordinary carriers, like silica gel, for example, commercial spherical or commercial broken silica gels, for example, Lichrosorb®,

Lichroprep®, Lichrospher® and Trisoperl®, and commercial polymer carriers, for example, Eupergit®, Fractogel®, especially Fractogel epoxy® and Fractoprep®, can be used. Silica gel is considered the preferred carrier material.

As an alternative, magnetic particles can also be used as carrier. These are preferably carrier materials with a magnetic core. This core is ordinarily enclosed by an inorganic oxide. The inorganic oxide is preferably silica gel. Examples of such magnetic carriers include *Magnesil*™ (Promega Corp., Madison, Wisconsin, US), *MagPrep*™ (Merck) and *AGOWAmag*™ (AGOWA GmbH, Berlin, Germany). Magnetic glass particles can also be used as magnetic carriers (for example, *MPG* (CPG Inc., Lincoln Park, New Jersey, US)), as well as magnetite-containing pigments (for example, *Microna Matte, Mica Black, Colorona Blackstar* (all Merck)). Nonporous magnetic particles (like MagPrep) are particularly suitable, since pore blocking cannot occur in them, by which the enzyme activity would deteriorate drastically.

The enzyme carrier ordinarily has the following properties: the particle size of the carrier is preferably 0.005-1 mm, even more preferably 0.01-0.5 mm. The pore diameter ordinarily lies in the range of 10-4000 nm, in which a pore diameter of 30-100 nm is particularly preferred. With a sufficiently large pore size, it can be ensured that the enzyme finds room on the carrier without activity loss. The particle surface is preferably 40-100 m^2/g and the pore volume is preferably chosen from a range between 0.5 and 3 mL/g. In many cases, a very large pore diameter of 2-20 μm can also be suitable.

The enzyme can be covalently or adsorptively coupled. A covalent coupling is generally preferred. Examples of covalent coupling include epoxidation, a carbodiimide method, silanization, a cyanogen bromide method, a glutardialdehyde crosslinking or dicresyl chloride method (see Biotransformations and Enzyme Reactions, Bommarius, A. S., Biotechnology (2nd Edition), Vol., 3, pp. 427-465, compiled by Stephanopoulos, G., VCH Weinheim, Germany, 1993, Walt, D.R., et al., Trends in Analytical Chemistry, Vol. 13, No. 10, 1994, N. H. Park, H. N. Chang; J. Ferment, Technol., Volume 57 (4), 310-316, 1979, M Puri et al.; Enz. Microb. Technol., 18, 281-285, 1996 and H-Y Tsen; J. Ferment. Technol., 62 (3), 263-267, 1984). To perform this method, it is necessary that the carrier be surface-modified with corresponding functional groups. The functional groups can be supplied to the carrier either by copolymerization with functional monomers or by a polymer-like conversion. Surface modification with amino residues, aldehyde groups, epoxy groups or diol modification is particularly preferred. The enzymes can then be covalently bonded to these groups.

Enzymatic hydrolysis occurs in an appropriate reactor. For continuous running of the method according to the invention, a commercial column is particularly suited. A column, as used for preparative HPLC, can be used on a small scale. The reactor, especially the column, should have a high hydrolytic efficiency. This can be quantified by the number of theoretical

plates. It is therefore advantageous that intensive contact of the raw material solution with the surface of the immobilizate occurs, in order to guarantee effective utilization of the enzyme and achieve high productivity. The mentioned preparative HPLC column satisfies these requirements, and is also equipped with the corresponding technique with peripherals (pumps, valves, control). It is also advantageous that detection techniques, like UV or RI detection techniques, have already been developed for this, so that, if desired, measurement and control of the degree of conversion of the reaction can be performed in automated fashion.

If magnetic carrier materials are used in the continuous procedure, tubular reactors are ordinarily used with the device that holds the magnetic particles stably in suspension, for example, electromagnetic coils that produce in the flow tube an essentially homogeneous magnetic field, whose lines of force performed parallel to the direction of flow (Helmholtz magnetic field). In such magnetically stabilized fluidized bed reactors (*MSFB*), much higher flow rates can be achieved than in conventional fluidized bed columns, which are also suitable for this purpose. This technology can also be used advantageously for catalysis reactions in viscous reaction media.

For batchwise performance of the method, an ordinary vessel is suitable, which is preferably equipped with an agitator. A round-bottomed flask with an agitator can be used on a small scale and a stirred vessel on a large scale.

The immobilizate is packed into the reactor in the usual manner before the reaction.

The rutinoside to be converted is fed to the reactor, for example, a column, like a fixed bed column, usually in the form of a solution or a suspension. If the reactor is a fixed bed reactor, the rutinoside solution should be fully solid-free. It is then advantageous to dissolve the rutinoside beforehand with the solvent in a tank, preferably during agitation and/or heating, in order to achieve optimal solubility. If necessary, prefiltration of the solution can also be carried out in order to eliminate possible solids. The solvent is preferably an aqueous system, in order to guarantee enzymatic activity and prevent possible denaturation. In order to guarantee solution of the rutinosides, other solvents can also be added. The method according to the invention is preferably performed in the presence of a solvent mixture consisting of water and at least one organic solvent.

The additionally present organic solvent or solvents include both organic solvents miscible with water and organic solvents immiscible with water.

Appropriate solvents for the method according to the invention include nitriles, like acetonitrile, amides, like dimethylformamide, esters, like acetates, especially methyl acetate or ethyl acetate, alcohols, like methanol or ethanol, ethers, like tetrahydrofuran or methyl-tert-butyl ether, and hydrocarbons, like toluene. The method according to the invention is preferably performed in the presence of one or more organic solvents acetates, methanol, ethanol,

methyl-tert-butyl ether or toluene. The method according to the invention, with particular preference, is performed in the presence of one or more acetates, especially in the presence of methyl acetate, in addition to water.

Appropriate volume ratios of water to organic solvents for the method according to the invention are ratios of 1:99-99:1. The method according to the invention is preferably performed with volume ratios of water to organic solvent of 20:80-80:20, especially with volume ratios of 50:50-70:30.

The amount of rutinoside in the solvent or solvent mixture for the method according to the invention is dependent on the solubility of rutinoside in the solvent or solvent mixture. For optimal performance of the method according to the invention, the rutinoside should be readily soluble. An undersaturated solution is therefore preferably used. Ordinarily, the amount of rutinoside in the solvent or solvent mixture is 0.001-5 g/L, preferably 0.05-2 g/L, even more preferably 0.1-1.5 g/L.

The ratio of rutinoside to immobilizate or enzyme depends on the lifetime of the enzyme of the column and its activity in immobilized form.

The reaction is ordinarily performed at a temperature of 15-80°C. A temperature of 30-60°C, especially a temperature of 40-50°C is particularly advantageous in order to counteract possible destruction of the enzyme and, at the same time, guarantee high solubility of rutinoside.

If the reaction temperature is too low, the reaction proceeds at an unsuitably slow reaction rate because of declining enzymatic activity. In addition, the solubility of rutinoside is reduced, so that needlessly high amounts of solvent are necessary. If the reaction temperature, on the other hand, is too high, the enzyme, which is a protein, is denatured and therefore deactivated.

If the method according to the invention is to be performed at elevated temperature, the reactor can be provided with a temperature control device. Ordinary temperature control devices include a heat coil system or double jacket. It is also advantageous, if the rutinoside being converted, and especially the rutinoside solution, is temperature-controlled before entering the reactor. For this purpose, it is common to feed the rutinoside solution from a temperature-controlled tank that is set at the temperature desired for the reaction. As an alternative, the solution to be supplied is passed through a heatable tube, in order to adjust the desired temperature before entering the reactor. Possible crystallization of rutinoside can also be avoided by temperature control.

Appropriate pH values for the method according to the invention are pH values between 3 and 8. The method according to the invention is preferably performed at pH values of 3-7, especially at pH values of 3-6. Additionally preferred pH values, however, can vary, depending

on the employed enzyme, within the stated limits. For example, pH values of 3.8-4.3 are quite extraordinarily preferred when the enzyme hesperidinase is used.

Preferably, the method is designed so that the pH value is set by means of a buffer system. In principal, all common buffer systems suitable for adjusting the aforementioned pH values can be used. However, aqueous citrate buffer is preferably used.

The rutinoside mixture, which can be present in the form of a solution or suspension, is introduced to the reactor containing the immobilizate, in order to perform the enzymatic hydrolysis. This reaction can be performed batchwise, i.e., discontinuously, or continuously.

If the reaction is performed batchwise, a rutinoside suspension is ordinarily introduced to the reactor. The degree of conversion is determined by the amount of rutinoside and immobilizate. Ordinarily, the ratio of rutinoside to immobilizate is 100:1 to 1:1000, preferably 10:1 to 1:100, more preferably 1:1 to 1:20. The ratio of immobilizate to total volume of the suspension is ordinarily 1:1000 to 1:1 preferably 1:100 to 1:2, even more preferably 1:50 to 1:5. The residence time in the reactor is normally 1 h to 10 days, preferably 8 h to 40 days, even more preferably 1-2 days.

If the reaction is performed continuously, a rutinoside solution is ordinarily permanently and continuously supplied with an appropriate pump through the reactor, preferably a column or MSFB reactor. By corresponding adjustment of the flow rate, any desired degree of conversion can be reached. Normally, a flow rate of 0.001-1 mm/s, with reference to the empty tube cross section of the column, is used.

The enzymatic activity in the system diminishes over time according to experience. It is therefore necessary that the immobilizate be entirely or partially renewed at regular intervals. In order to compensate for activity loss of the enzyme, it is advantageous to evaluate the degree of conversion by UV or RI detection, so that during a change in composition, control of change can be counteracted via the pump delivery.

After discharge of the reaction solution, the obtained product can be separated. After the reaction is completed, the reaction mixture consists mainly of solvent, unconverted rutinoside, rhamnose, the desired monoglycosidated flavonoid and possibly other additives, like buffers. Ordinarily, the monoglycosidated flavonoid precipitates as soon as the solubility limit is reached and is gradually enriched as solid.

In a batchwise procedure with magnetic carrier materials, the immobilized enzyme can be separated after the reaction is completed in simple fashion, by means of a magnetic separating device, from the product suspension. On a laboratory scale, a strong permanent magnet in the form of a plate can be used. However, there are also larger separators that have been developed for different industrial applications and generally operate according to the HGMS principle (high gradient magnetic separation). This type of installation can consist of an upright flow tube,

containing a packing of fine stainless steel wires. By means of appropriately arranged electromagnetic coils, high magnetic field gradients are produced along the wires and, because of this, very effective separation of even the smallest particles in the nanometer range is achieved. If the magnetic particles are superparamagnetic, i.e., have no remanent magnetization in the absence of an external magnetic field, after switching off the magnetic field, they can be fully separated again from the reactor without problem by repeated rinsing with water.

Isolation of the desired reaction product occurs according to the usual methods in a usual workup.

The product is preferably precipitated during concentration. If the solvent is a solvent mixture, containing at least one organic solvent, it is preferred that the organic solvent be distilled off under reduced pressure. The crystallizing monoglycosidated flavonoid is ordinarily separated from the remaining reaction mixture by a solid/liquid separation, like suction filtration or filtration under reduced pressure, or centrifuging of the precipitated crystals. The solid is then washed, preferably with water, and then dried.

As an alternative, the entire reactor contents can initially be filtered off. The filter cake containing the product is then treated with a solvent or buffer-solvent mixture in which the product is soluble. The reaction product is extracted from the filter cake.

In a batchwise procedure, the catalyst insoluble in this mixture and the immobilizate remain. It is therefore necessary that the solvent or buffer-solvent mixture not have an adverse effect on the enzyme. It has been found that the immobilized enzyme, for example, naringinase or hesperidinase, has no or only a fraction of the original activity in certain solvent-buffer mixtures or moderately alkaline conditions, but that the activity can be almost fully restored, if the enzyme is carefully rinsed with a buffer in the pH range 4-6; a temporary loss of activity is therefore involved, not an irreversible denaturation of the enzyme.

Extractants very suited for this procedure are tetrahydrofuran-buffer mixtures, preferably with a tetrahydrofuran fraction of 10-25%, especially at a slightly elevated temperature. Other appropriate extracting components include 1-propanol, 2-propanol, 1,4-dioxane and methyl acetate. The product can be very easily recovered from the extract by distilling the solvent off under reduced pressure, and then cooling the aqueous product solution to 0-10°C. The reaction product crystallizes from the mother liquor in very high purity.

As an alternative to solvent/buffer mixtures, a dilute ammonia or soda solution can also be used as extractant, since the reaction product has phenolic OH groups that are already deprotonated in a weakly basic medium; the anion of the reaction product is comparatively readily soluble, but also very sensitive to oxidation, which makes itself noticeable by gradual discoloration of the extract from yellow to brown. In this variant, the operation should therefore

be accomplished quickly, i.e., the extraction should preferably be concluded within 10 min to 6 h, preferably 20 min to 2 h. A protective gas atmosphere is preferably used.

By treatment with weakly basic extractants, like aqueous solutions of alkali or ammonium salts of acetic acid, oxalic acid, citric acid, phosphoric acid, boric acid or carbonic acid, or aqueous solutions of alkylamines, piperidine or pyridine, enzyme activity is not lost either. By acidification of the extract and cooling to 0-10°C, the reaction product can be precipitated again.

The purity of the obtained monoglycosidated flavonoid during use of pure rutinoside is normally greater than 94%. For further purification, the end product can be recrystallized, for example, from appropriate solvents, for example, from water or solvent mixtures consisting of toluene or methanol or water and methyl acetate.

The forming amount of solvent after the reaction is preferably recovered in order to guarantee economic efficiency of the method according to the invention. This recirculation ordinarily occurs continuously and is automated. Commercial evaporator units with corresponding control work for this. The solvent being employed is a solvent mixture of water and at least one organic solvent, in which case it is generally not possible to reuse the distillate immediately for the method, since the solvent ratio is altered by distillation of the organic solvent. By automatic quality measurement and correction, the desired solvent ratio can then be restored and the solvent therefore recycled.

Membrane processes or nanofiltration can also be carried out during concentration. In these methods, the solvent mixture is separated without a change in composition.

The following examples will explain the present invention. However, they are in no way to be viewed as limiting.

Example 1

Immobilization of the enzyme hesperidinase on a silica gel carrier.

1) Carrier surface conditioning for immobilization

1.1) Properties of the carrier material

Silica gel LiCrospher

Diameter = 15-40 μm

Pore diameter = 300 Å

Particle surface = 80 m^2/g

Pore volume = 0.73 mgL/g

Density = 2 g/mL

1.2) Silica gel activation

250 g silica gel are mixed with sufficient HCl (7%) in a 1-L bottle and allowed to stand overnight, in order to moisten the silica gel.

The silica gel suspension is then washed chloride-free with demineralized water. For this purpose, after each washing, the supernatant must be tested with nitric acid and silver nitrate. Washing is conducted on a roughly 24 cm diameter ceramic funnel, owing to the properties of the silica gel particles.

1.3) Surface modification with amino groups

The acid-treated silica gel is mixed in a 2 liter, three-necked flask equipped with a reflux condenser and dropping funnel, together with sufficient water, so that it is stirrable. At room temperature with good agitation, 1 mmol/g carrier of 3-aminopropyltrimethoxysilane (for 250 g silica gel, 135 mL of solution is required) is added dropwise at a rate of about 5 drops/sec to the silica gel suspension. The suspension is then agitated for 2 h at 90°C. The suspension is then cooled with ice.

The supernatant must be checked by pH value measurement for possible residues of 3-aminopropyltrimethoxysilane. The bead suspension is washed with demineralized water, until the pH value remains constant.

1.4) Glutardialdehyde demonstration

Glutardialdehyde (GDA) in a concentration of 1 mmol/g carrier (for 250 g carrier, 13 mL 50% GDA solution is required) is added to the obtained silica gel suspension. The suspension (plus some water) is rolled for 2 h at room temperature in a 1-L bottle. Initially, the suspension is yellow and, at the end, becomes dark red.

The supernatant from each washing is then checked by a precipitation reaction with dinitrophenylhydrazine for glutardialdehyde residues. The suspension is carefully washed, until a test is negative.

2) Immobilization

2.1) Hesperidinase

First protein addition

3.8 g hesperidinase are agitated in 500 mL citrate/phosphate buffer (pH = 6.0). For better dissolution, 300 μL surfactant (Tween 20) are added. The enzyme solution is then filtered.

About 230 g of the silica gel suspension obtained under 1.4 is mixed in a 1-L bottle with the enzyme solution. The enzyme-carrier suspension is then rolled for about 40 h at room temperature.

Second protein addition

About 0.76 g hesperidinase (Amano) is agitated in 120 mL citrate/phosphate buffer (pH = 6.0) with 60 μL surfactant and later filtered.

The enzyme solution is poured into the aforementioned 1-L bottle and the enzyme solution rolled at room temperature.

2.2) BSA (for the separation test)

0.3 Biomex BSA (bovine serum albumin powder) is agitated in 100 mL citrate/phosphate buffer (pH = 6.0). About 20 g of the silica gel suspension obtained under 1.4 are mixed in a 0.5 L flask with the protein solution and 60 μL ProClin300 are added.

3) Protein amounts and activity determination

3.1) Protein amount (mg protein/mL)

The protein content of a solution is determined by the Bradford test. The standard assay is performed. 20 μL of sample are mixed in 1 mL Bradford dye reagent (1:5 diluted) and measured after 15 min in a photometer at 595 nm.

At very low protein concentrations, the microassay must be conducted. 0.8 mL of sample is mixed in 0.2 mL Bradford dye reagent (concentrated) and measured after 15 min in the photometer at 595 nm.

3.2) Activity

The activity of the solution is measured by the reaction with a substitute substrate. For each sample, one uses:

88 μL citrate/phosphate buffer (pH = 4.0)
100 μL sample
20 μL sample
20 μL substitute substrate: p-nitrophenyl-α-L-rhamnoside (rhanmosidase activity)
p-nitrophenol-α-L-glucoside (glucosidase activity)

This 1-mL solution is mixed in an Eppendorf reaction vessel. After an incubation time of 2 and 5 min at 40°C in a shaker, 100 μL of each reaction mixture are mixed with 1 mL of 1 M soda solution. The concentration of p-nitrophenol is then measured in the photometer at 400 nm. The activity is calculated by the concentration change of p-nitrophenol over time.

The activity of an enzyme is stated in units (U) (= μmol converted substrate per minute).

4) Results

PROTEIN CONTENT and ACTIVITY VALUES*

(FREE HESPERIDINASE)

1 Probe[1]	2 Proteinmenge (Standard-Assay)		3 Aktivität (Ersatzsubstrat)		
	mg	mg/ml Probe 1	U	U/ml Probe 1	U/mg Protein
Hesp0	740	1,48	48000	96	65
1	15	0,03	7	0,014	0,47
2	20	0,04	4	0,008	0,2
Hesp1	326	2,72	11400	95	35
3	56	0,09	1450	2,33	26
4	28	0,045	361	0,58	13
5	22	0,035	95	0,15	4,3
6	20,5	0,033	11	0,018	0,54

[1] HespO: first protein addition
Hesp1: second protein addition
Samples 1-6: supernatant

Key: 1 Sample[1]
2 Protein amount (standard assay)
3 Activity (substituted substrate)

PROTEIN CONTENT and ACTIVITY VALUES

(IMMOBILIZED HESPERIDINASE)

* Protein content in carrier ≅ 1.045 g bound protein
* 4.7 mg protein/g carrier on the carrier
* 2% added protein not bonded

Example 2

Preparation of isoquercetin from rutin by enzymatic-hydrolysis using an immobilizate.

* [In the original document, commas in numbers represent decimals.]

In a heatable 4.5 m^3 stir vessel (1), 3200 L demineralized water and 800 L 1-propanol are introduced. The mixture is heated to about 50-60°C via steam feed (2). Under strong agitation, 8000 g rutin, DAB is added to the solution. The mixture is agitated, until the rutin has completely dissolved. The pH value is checked via a metering pump and a pH meter (3a) mounted in the line, and, if necessary, set at pH 4.0-4.5 (with H_3PO_4 and NaOH). A sample can also be taken by a manual valve (4) for control and concentration determination.

To start the reaction, the solution is supplied via a bag filter (5) and cartridge filter (6) to the piston metering pump (7). The bag filter then has the task of retaining larger amounts of undissolved components, whereas the cartridge filter cleans the solution with a fineness of 0.2 µm.

The piston metering pump (7) conveys the solution for a heatable tube, which sets the solution via a thermometer at a temperature of 40°C at the inlet to the column, with a flow rate of 1 L/min in column (9) (100 × 400 mm). The column contains 1.5 kg immobilizate. Since the electrically heated tube cannot cool the solution, the temperature in this third vessel (1) must be chosen, so that cooling on the way to the pump leads to 40°C at maximum pump flow.

A sample can be taken from the solution after passing through the column via a manual valve (10), so that the temperature and degree of conversion of the reaction can be measured again offline. If the measured degree of conversion is less than required, the flow rate of the pump is correspondingly reduced.

After passing through the column, the reaction is fully completed, so that the solution can be fed to trapping vessel (11). There the solution is concentrated via a condenser (12) to about 10-20% of the volume. The content of propanol is significantly reduced by this, so that the solubility of isoquercetin drops sharply. By subsequent cooling, the solubility drops farther, so that the product precipitates and can be separated in the bag filter (13). From there, it is transferred to a drying cabinet (14) for further drying. The mother liquor and the distilled condensate are returned together for recharging in the stir vessel (1).

<u>Example 3</u>

1. Modification of silica gel particles without the aldehyde groups and immobilization of naringinase on these particles

250 g silica gel (for example, LiChrospher SI 300, Merck, Darmstadt) was transferred to a sealable vessel with 400 mL 10% HCl, degassed for 10 min with ultrasound and allowed to stand for 24 h at room temperature. The silica gel was then filtered off and washed with several liters of demineralized water, until the pH value > 4.5 and no chloride ions were detectable in the filtrate (spot reaction with an acetic acid $AgNO_3$ solution).

The acid-treated moist silica gel was then transferred to a 4-L, three-necked flask equipped with a KPG stirrer, reflux condenser and 100-mL dropping funnel, and suspended with 3 L demineralized water. During agitation, 100 mL aminopropyltrimethoxysilane (ABCR, Karlsruhe) were added via the dropping funnel over 15 min. The suspension was then heated and agitated for 90 min at 90°C. The cooled suspension was filtered and washed eight times with 1-L demineralized water each time.

The amino-activated silica gel was suspended in a 4-L, three-necked flask equipped with a KPG agitator and 100-mL dropping funnel, in 3-L water degassed with ultrasound; the pH value was reduced with a few drops of 2 M acetic acid to 8.0. 100 mL 50% glutardialdehyde solution (Merck, Darmstadt) were then added dropwise over 1 h and the suspension agitated another 2.5 h at room temperature. The activated silica gel was filtered again and washed with ice-cold demineralized water, until glutaraldehyde was no longer detectable in the wash water (spot reaction with a 2,4-dinitrophenolhydrazine solution in sulfuric acid).

The silica gel modified with aldehyde groups was suspended in 500 mL demineralized water in a 4-L flask by agitation with a KPG agitator. 13 g naringinase (Sigma, Deisenhofen) were dissolved in 2.5 L 0.25 M phosphate buffer, pH 8.0. The enzyme solution was introduced to the silica gel suspension and agitated for 96 h at room temperature. The immobilizate was then filtered off and initially washed several times with 0.2 M sodium chloride solution, then 50 mM citrate buffer, pH 4.0. The rhamnoside activity of the immobilizate was determined with p-nitrophenyl-L-α-rhamnopyranosie (Sigma, Deisenhofen) as substrate according to the Kurosawa method; it amounted to 120 U/g.

2. Immobilization of hesperidinase on Eupergit™C

50 g Eupergit (Röhm, Weiterstadt) were mixed in a screwable 500-mL glass bottle with 300 mL 0.8 M potassium phosphate buffer, pH 8.5, and allowed to stand for 30 min. 5.0 g hesperidinase (Amano) were then added and the charge moved on a roll mixer for 120 h at room temperature. The Eupergit was filtered off with a glass frit and initially washed several times with 0.2 M sodium chloride solution, then twice with 1-L 0.1 M citrate buffer each time, pH 4.0. The rhamnoside activity of the immobilizate was determined with p-nitrophenyl-L-α-rhamnopyranoside (Sigma, Deisenhofen) as substrate according to the Kurosawa method; it amounted to 15 U/g, referred to dry immobilizate, or 4.2 U/g with reference to moist immobilizate.

3. Conversion of rutin to isoquercetin with hesperidinase immobilized on Eupergit in a stirred vessel reactor and subsequent extraction of the product with a tetrahydrofuran-buffer mixture

1000 mL 50 mM citrate buffer, pH 4.0, 100 g (moist weight) naringinase immobilized on Eupergit, with an activity of 4.2 U/g, as well as 10 g rutin (Merck, Darmstadt), were agitated at 40°C with a KPG agitator in a 2000-mL round-bottomed flask; the degree of conversion was continuously determined by HPLC analysis. After a total of 96 h, the reactor contents were filtered off via a Büchner funnel. The filter cake was returned to the round-bottomed flask and agitated for 30 min in a mixture of 400 mL 50 mM citrate buffer, pH 4.0, and 100 mL tetrahydrofuran at 40°C, during which most of the isoquercetin dissolved. The mixture was then filtered and the filter cake extracted again for 30 min with 500 mL buffer-tetrahydrofuran mixture. After filtration, the two isoquercetin extracts were combined with a first filtrate and the tetrahydrofuran removed with a rotary evaporator. In order to fully precipitate the product, the aqueous isoquercetin solution was cooled to 4°C. After filtration and drying in a desiccator, a yield of 5.8 g of product was obtained, which consisted of 98% isoquercetin and 2% rutin.

The moist Eupergit was washed once with a cold tetrahydrofuran-buffer mixture, then repeatedly with 50 mM citrate buffer, pH 4.0, until the odor of tetrahydrofuran was only still weakly detectable. The activity of the enzyme was still 3.6 U/g, which corresponds to an activity loss of 14%.

4. Conversion of rutin to isoquercetin with hesperidinase immobilized on Eupergit in a stirred vessel reactor and subsequent extraction of the product with an alkaline buffer solution

100 mL 50 mM citrate buffer, pH 4.0, 100 g naringinase immbolized on Eupergit, with an activity of 4.2 U/g (moist weight), as well as 10 g rutin (Merck, Darmstadt), were agitated at 40°C with a KPG agitator in a 2000 mL round-bottomed flask; the degree of conversion was continuously determined by HPLC analysis. After a total of 96 h, the reactor contents were filtered off via a Büchner funnel. The moist filter cake was returned to the round-bottomed flask and agitated at room temperature for 5 min in 300 mL of 50 mM sodium carbonate buffer, pH 10.0, during which part of the isoquercetin dissolved with an intense yellow color. The suspension was filtered and a filter cake immediately extracted again with carbonate buffer. After a total of 7 extraction cycles, the Eupergit was largely colorless and the isoquercetin almost fully dissolved. The extracts were combined, carefully acetified with dilute hydrochloric acid, until the pH was about 3, and then cooled to 4°C. After filtration and drying in the desiccator, a yield of 4.9 g of product was obtained, which consisted of 98% isoquercetin and 2% rutin.

The moist Eupergit was washed twice with 50 mM citrate buffer, and was then ready for use for additional conversions. The activity of the enzyme was still 3.9 U/g, which corresponds to an activity loss of 7%.

Example 4

1. Modification of magnetic silica particles with aldehyde groups and immobilization of naringinase on these particles

A suspension of 30 g magnetic silica particles (MagPrep, Merck, Darmstadt) in 600 mL water was introduced to a 1-L, three-necked flask equipped with a KPG agitator, dropping funnel and reflux condenser. A mixture of 20 mL aminopropyltriethoxysilane (ABCR, Karlsruhe) and 20 mL isopropanol was added dropwise over 30 min during agitation. The mixture was then heated to 85°C and agitated for 1 h at this temperature. After cooling, the suspension was transferred to a beaker, the particles collected with a strong permanent magnet on the bottom of the vessel and the supernatant decanted. The particles were repeatedly washed with demineralized water, until the pH value of the wash water remained constant. The particles were then resuspended in 600 mL water and the pH value set at a value of about 8 with a few drops of acetic acid; after addition of 24 mL 50% glutardialdehyde solution, the suspension was agitated for 4 h at room temperature and the particles washed with demineralized water, until glutaraldehyde was no longer detectable in the wash water (spot reaction with 2,4-dinitrophenylhydrazine solution in sulfuric acid).

The aldehyde-derived particles were resuspended in a 1-L round-bottomed flask in 600 mL 0.2 M potassium phosphate buffer, pH 9. After addition of a solution of 1 g naringinase (Sigma, Deisenhofen) in 100 mL 50 mM sodium chloride solution, the mixture was agitated with a KPG agitator for 2 days at room temperature. The particles were then separated with a permanent magnet and initially washed repeatedly with 0.2 M sodium chloride solution, then with 50 mM citrate buffer, pH 4.0. The rhamnosidase activity of the immobilizate was determined with p-nitrophenyl-L-α-rhamnopyranoside (Sigma, Deisenhofen) as substrate according to the Kurosawa method (Kurosawa, Ikeda, Egami, *J. Biochem* 73, 31-37 (1973); α-L-rhamnosidases of the liver of *Turbo cornutus* and *Aspergillus niger*); it amounted to 162 U/g.

2. Modification of magnetic silica particles with epoxy groups and immobilization of naringinase on these particles

A suspension of 30 g magnetic silica particles (MagPrep, Merck, Darmstadt) in 600 mL 50 mM sodium acetate solution was introduced to a 1-L, three-necked flask equipped with a KPG agitator, dropping funnel and reflux condenser. A mixture of 20 mL (3-glycidoxypropyl)trimethoxysilane (ABCR, Karlsruhe) and 20 mL isopropanol were added dropwise over 30 min during agitation, and the mixture heated to 85°C and agitated for 1 h at this temperature. After cooling, the suspension was transferred to a beaker, the particles collected

with a strong permanent magnet on the bottom of the vessel and the supernatant decanted. The particles were repeatedly washed with demineralized water, until the pH value of the wash water remained constant. For quantitative determination of epoxy groups, a sample of about 0.5 g of the material was repeatedly washed with methanol and then dried at about 70°C in a drying cabinet to constant weight. Determination of the epoxy groups according to the Pribyl method (Pribyl, *Fresenius Z. Anal. Chem.* 303, 113-116 (1980): Determination of epoxide groups in modified chromatographic sorbents and gels) gave 250 μmol/g.

150 mL of a 20% (w/v) suspension of epoxy-derived magnetic particles was mixed in a 1-L round-bottomed flask with 350 mL 1 M potassium phosphate buffer, pH 9.0. After addition of the solution to 1.5 g naringinase (Sigma, Deisenhofen) in 15 mL 50 mM sodium chloride solution, the mixture was agitated for 16 h at 40°C with a KPG agitator. The particles were then separated with a permanent magnet and washed repeatedly with 0.2 M sodium chloride solution first, then with 50 mM citrate buffer at pH 4.0. The rhamnosidase activity of the immobilizate was determined with p-nitrophenyl-L-α-rhamnopyranoside (Sigma, Desienhofen) as substrate according to the Kurosawa method; it amounted to 102 U/g.

3. Modification of magnetic silica particles with carboxyl groups and immobilization of naringinase on these particles

A suspension of 30 g magnetic silica particles (MagPrep, Merck, Darmstadt) in 600 mL water was introduced to a 1-L, three-necked flask equipped with a KPG agitator, dropping funnel and reflux condenser. A mixture of 28 mL 3-(triethoxysilyl)propylsuccinyl anhydride (ABCR, Karslruhe) and 28 mL isopropanol was added dropwise over 30 min with agitation, and the pH value of the reaction mixture then set a 9.0 by dropwise addition of 10% NaOH. The mixture was heated to 80°C and agitated at this temperature for 2 h. The pH was checked at regular intervals and corrected, if necessary, by addition of alkali. After cooling, the suspension was transferred to a beaker, the particles collected with a strong permanent magnet on the bottom of the vessel and the supernatant decanted. The particles were washed three times with demineralized water, once with a 2 M acetic acid solution, and then repeatedly with demineralized water, until the pH value of the wash water remained constant.

150 mL of a 20% (w/v) suspension of carboxyl-derived magnetic particles was mixed in a 1-L round-bottomed flask with 300 mL 0.4 M potassium phosphate buffer, pH 5.0, and a solution of 1.5 g naringinase (Sigma, Deisenhofen) in 150 mL 50 mM sodium chloride solution. After addition of 8 mL of a 1% (w/v) solution of EDC (N-ethyl-N'(3-dimethylaminopropyl)carbodiimide hydrochloride, Merck, Darmstadt) in water, the mixture was agitated for 20 h at room temperature. The particles were then separated with a permanent magnet and repeatedly washed first with 0.2 M sodium chloride solution, then with 50 mM

citrate buffer, pH 4.0. The rhamnosidase activity of the immobilizate was determined with p-nitrophenyl-L-α-rhamnopyranoside (Sigma, Desienhofen) as substrate according to the Kurosawa method; it amounted to 71 U/g.

4. Modification of magnetic mica pigments with aldehyde groups and immobilization of hesperidinase on these particles

A suspension of 30 g magnetic mica pigments ("Colorona Blackstar Green", Merck, Darmstadt) in 300 mL water was introduced to a 1-L, three-necked flask equipped with a KPG agitator, dropping funnel and reflux condenser. A mixture or 20 mL aminopropyltriethoxysilane (ABCR, Karlsruhe) and 20 mL isopropanol was added dropwise over 30 min during agitation. The mixture was then heated to 85°C and agitated for 1 h at this temperature. After cooling, the suspension was transferred to a beaker, the particles collected on the bottom of the vessel with a permanent magnet and the supernatant decanted. The particles were washed repeatedly with demineralized water, until the pH value of the wash water remained constant. The particles were then resuspended in 300 mL water and the pH value set at about 8 with a few drops of acetic acid; after addition of 25 mL 50% glutardialdehyde solution, the suspension was agitated for 4 h at room temperature and the particles washed with demineralized water, until glutaraldehyde could no longer be detected in the wash water (spot reaction with 2,4-dinitrophenylhydrazine solution in sulfuric acid).

30 g aldehyde-derived mica pigments "Colorona Blackstar Green" was resuspended in a 1-L round-bottomed flask in 300 mL 0.2 M potassium buffer, pH 7.5. After addition of a solution of 1 g hesperidinase (Amano) in 20 mL 0.2 M potassium phosphate buffer, pH 7.5, the mixture was agitated for 3 days at room temperature with a KPG agitator. The particles were then separated with a permanent magnet and washed repeatedly, first with 0.2 M sodium chloride solution, then with 50 mM citrate buffer, pH 4.0. The rhamnosidase activity of the immobilizate was determined with p-nitrophenyl-L-α-rhamnopyranoside (Sigman, Deisenhofen) as substrate according to the Kurosawa method; it amounted to 10 U/g.

5. Conversion of rutin to isoquercetin with immobilized naringinase in a stirred vessel reactor and separation of the magnetic biocatalyst with a permanent magnet

400 mL 50 mM citrate buffer, pH 5.0, 20 g naringinase immobilized on magnetic silica particles with an activity of 102 U/g and 10 g rutin (Merck, Darmstadt) were mixed with each other at 40°C in a 500 mL double-jacket stirred reactor; the degree of conversion was determined at intervals by HPLC analysis. After 24 h, the reactor contents were pumped into a beaker and the catalyst collected on the bottom of the vessel with a plate magnet (Bakker, 200 mT). The supernatant was immediately filtered by suction with a pump and the magnetic particles washed

repeatedly with 100 mL buffer each time in order to rinse out the last residues of adhering solid isoquercetin. The collected isoquercetin was filtered off, washed several times with small batches of ice water and dried in a desiccator. The yield was 6.5 g. HPLC analysis gave a composition of 96% isoquercetin, 2% quercetin and 2% rutin. The activity of the immobilizate after conversion was still 92 U/g. This corresponds to an activity loss of 10%.

6. Conversion of rutin to isoquercetin with immobilized naringinase in a stirred vessel reactor and separation of the magnetic biocatalyst with an electromagnetic separator (Figure 1)

300 mL 50 mM citrate buffer, pH 5.0, 10 g naringinase immobilized on magnetic silica particles with an activity of 102 U/g and 5 g rutin (Merck, Darmstadt) were mixed with each other at 40°C in a 500 mL double-jacket stirred reactor; the degree of conversion was continuously determined by HPLC analysis. After 24 h, the reactor contents were passed through an electromagnetic HGMS installation, by means of a peristaltic pump with a flow rate of 25 mL/min, so that the magnetic particles were fully deposited on the wired matrix (technical data of the separation unit: glass tube with an inside diameter of 20 mm and length of 200 mm, empty volume 65 mL, weight of the wire packing made of SS alloy 15 g, 4 coils connected in series, current intensity 6 A, magnetic field intensity of the Helmholtz field 25 mT). 100 mL citrate buffer was then pumped through the column twice with the magnetic field engaged in order to rinse out the magnetic particles. The combined product suspensions were filtered, the isoquercetin washed with ice water and dried in the desiccator. The yield was 3.1 g. HPLC analysis showed a composition of 97% isoquercetin, 2% quercetin and 1% rutin.

To recover the catalyst, 100 mL citrate buffer, pH 5.0, was circulated through the separation unit at a flow rate of 100 mL/min with the magnetic field disengaged for 10 min, during which the flow direction was changed several times. The catalyst suspension was then pumped back into the stirred vessel and the remaining amount of catalyst in the separator was rinsed twice with 100 mL citric buffer each time. The activity of the immobilizate after conversion was still 94 U/g. This corresponds to an activity loss of 8%.

7. Conversion of rutin to isoquercetin with hesperidinase immobilized on mica particles in a stirred vessel reactor and separation of the magnetic biocatalyst with a plate magnet

400 mL 50 mM citrate buffer, pH 5.0, 30 g hesperidinase immobilized on "Colorona Blackstar" with an activity of 10 U/g and 5 g rutin (Merck, Darmstadt) were mixed with each other 40°C in a 500 mL double-jacket stirred reactor; the degree of conversion was continuously determined by HPLC analysis. After 24 h, the reactor contents were pumped into a beaker and the catalyst collected with a plate magnet (Bakker, 200 mT) on the bottom of the vessel. The supernatant was immediately filtered by suction with a pump and the magnetic particles washed

repeatedly with 100 mL buffer in order to rinse out the last residues of adhering solid isoquercetin. The collected isoquercetin was filtered off, washed several times with small batches of ice water and dried in the desiccator. The yield was 3.3 g. HPLC analysis gave a composition of 96% quercetin and 4% rutin.

The activity of the immobilizate after conversion was still 9.7 U/g. This corresponds to an activity loss of 3%.

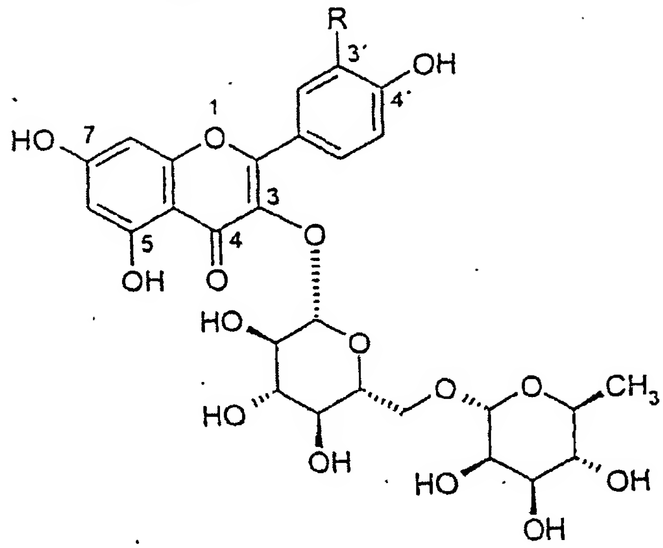
8. Conversion of rutin to isoquercetin with naringinase immobilized on magnetic silica gel particles in an MSFB reactor

A mixture of 5 g rutin, 900 mL 50 mM citrate buffer, pH 5.0, and 100 mL methyl acetate were agitated at 40°C in a supply vessel, until a roughly homogeneous suspension had formed without larger agglomerates. The reactor was not temperature-controlled. The methyl acetate was added, in order to increase the solubility and final dissolution rate at room temperature, as well as to prevent formation of quercetin. A suspension of 6 g naringinase immobilized on magnetic silica particles, with an activity of 162 U/g in 60 mL 50 mM citrate buffer, pH 5.0, was supplied with the magnetic field disengaged, using the pump, into the tube of the MSFB reactor. A 20 mT strong magnetic field was then established and fresh citrate buffer was introduced initially from the bottom with a precisely meterable piston pump with a flow rate of 5 mL/min, until the particles had stabilized in the magnetic field. The rutin suspension was then pumped through the MSFB reactor at room temperature over 3.5 h. The methyl acetate was initially eliminated in the rotary evaporator from the product mixture; the product was then filtered off, washed several times with ice water and dried in the desiccator. The product yield was 2.9 g; the product consisted of 86% isoquercetin and 14% rutin.

Claims

1. Method for the production of monoglycosidated flavonoids by enzymatic hydrolysis of rutinosides, characterized by the fact that an enzyme immobilized on a carrier is used for enzymatic hydrolysis.

2. Method according to Claim 1, characterized by the fact that a rutinoside of formula (A)



(A)

in which R denotes H, OH or OCH_3,

is used.

3. Method according to Claim 1 or 2, characterized by the fact that rutin is used in the form of rutinoside.

4. Method according to Claims 1-3, characterized by the fact that an α-L-rhamnosidase is used as enzyme.

5. Method according to Claims 1-4, characterized by the fact that hersperidinase is used as enzyme.

6. Method according to Claims 1-5, characterized by the fact that the enzyme is immobilized on silica gel.

7. Method according to Claims 1-5, characterized by the fact that enzymatic hydrolysis is performed in the presence of a solvent mixture of water and at least one organic solvent.

8. Method according to Claims 1-7, characterized by the fact that the reaction is performed at a reaction temperature of 15-80°C.

9. Method according to Claims 1-8, characterized by the fact that the reaction is performed at a pH value of 3-8.

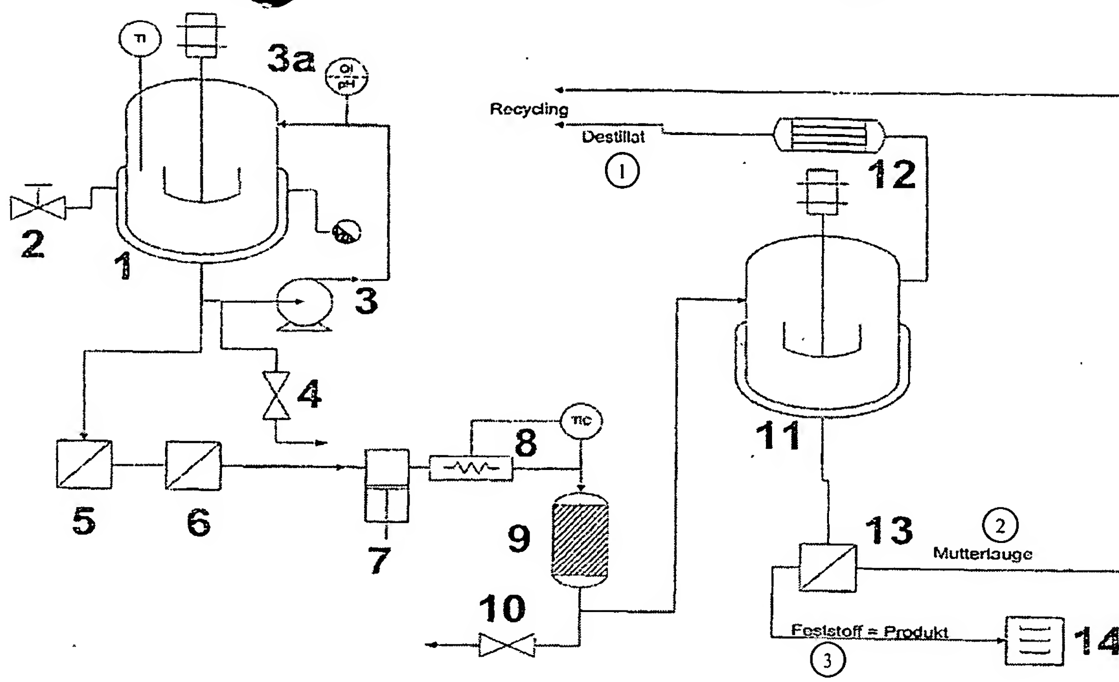


Figure 1

Key: 1 Distillate
2 Mother liquor
3 Solid = Product

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/01447

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12P19/02 C12P19/60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 971 812 A (NATIONAL SCIENCE COUNCIL) 20 November 1990 (1990-11-20) Siehe insbesondere Spalten 1 und 2 ---	1-9
X	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; TURECEK, P. L. ET AL: "Applications of enzyme immobilization in the analysis of naturally occurring compounds, for example,.alpha.-L-rhamnosides" retrieved from STN Database accession no. 109:34782 XP002168065 abstract & SCI. PHARM. (1987), 55(4), 275-83 , --- -/--	1-9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
22 May 2001	05/06/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Douschan, K

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 01/01447

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; 1989 TSEN H-Y ET AL: "FIBER ENTRAPMENT OF NARINGINASE FROM PENICILLIUM-SP AND APPLICATION TO FRUIT JUICE DEBITTERING" Database accession no. PREV198987114493 XP002168066 abstract & JOURNAL OF FERMENTATION AND BIOENGINEERING, vol. 67, no. 3, 1989, pages 186-189, ISSN: 0922-338X	1-9
X	DATABASE BIOSIS 'Online! BIOSCIENCES INFORMATION SERVICE, PHILADELPHIA, PA, US; January 1998 (1998-01) ELLENRIEDER G ET AL: "Hydrolysis of supersaturated naringin solutions by free and immobilized naringinase." Database accession no. PREV199800134334 XP002168067 abstract & BIOTECHNOLOGY TECHNIQUES, vol. 12, no. 1, January 1998 (1998-01), pages 63-65, ISSN: 0951-208X	1-9
Y	DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; TURECEK, P. ET AL: "Simple enzyme reactors suitable for the byproduct-free preparation of the aglycones of naturally occurring glycosides under mild conditions" retrieved from STN Database accession no. 105:95983 XP002168068 abstract & APPL. BIOCHEM. BIOTECHNOL. (1986), 13(1), 1-13 ,	1-9
Y	EP 0 317 033 A (UNILEVER PLC) 24 May 1989 (1989-05-24) cited in the application the whole document	1-9
Y	US 5 641 659 A (HOECHST AG) 24 June 1997 (1997-06-24) column 7	1-9

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 01/01447

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
US 4971812	A	20-11-1990	NONE			
EP 0317033	A	24-05-1989	AT	92109	T	15-08-1993
			CA	1333780	A	03-01-1995
			DE	3882655	A	02-09-1993
			DE	3882655	T	18-11-1993
			WO	8904870	A	01-06-1989
			ES	2058241	T	01-11-1994
			JP	2502248	T	26-07-1990
			MX	170209	B	11-08-1993
			PT	89040	A,B	01-12-1988
			US	5077206	A	31-12-1991
US 5641659	A	24-06-1997	CZ	9302543	A	15-06-1994
			EP	0599159	A	01-06-1994
			JP	6209773	A	02-08-1994
			SG	54274	A	16-11-1998
			US	5468625	A	21-11-1995